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BLOOD TYPING IN JAPANESE QUAIL: LINE DIFFERENCES BY HEMOLYSIS AND LYMPHOCYTE CYTOTOXICITY

by

© PATRICIA ANN KRUK

A THESIS

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Date August 26th 1983

Abstract

Discrimination was achieved at the level of the erythrocytes and lymphocytes between established lines of *Coturnix coturnix japonica* previously shown to be internally histocompatible within lines and histoincompatible between lines by skin grafting experiments.

Twenty four lectins failed to show differentiation of the red blood cells (RBC) of these quail lines in agglutination tests. The lectins either agglutinated all RBC's or they did not agglutinate any RBC's.

Anti-chicken lymphocyte and RBC B blood group 2/2, 14/14, 15/15, 19/19, and 21/21 monoclonal antibodies failed to react with the lymphocytes of five lines of Japanese quail.

Alloantisera were developed against five quail lines. Much of the extensive cross-reactivity demonstrated in the RBC hemolysis tests was lost following absorption of the antisera with RBC's of the remaining histoincompatible lines. Line-specific RBC activity remained, but at low titers.

After similar absorption with whole blood from the histoincompatible lines, line-lymphocyte specific alloantisera were developed. Contrary to earlier reports, a rapid, reproducible, accurate, and extremely sensitive complement-mediated lymphocyte cytotoxicity test was developed using quail lymphocytes, quail alloantisera, and

rabbit complement. This technique proved to be a more desirable form for differentiating between quail lines than conventional typing methods using the less sensitive erythrocyte tests. The conditions of and the results of such tests are reported herein.

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I take pleasure in acknowledging the support and guidance of my supervisor, Dr. Royal F. Ruth, whose wisdom and experience facilitated the completion of this thesis. I am grateful to Dr. Soledad R. Sawada for her helpful discussions and assistance. I would like to thank Makoto Mizutani (Nippon Institute for Biological Science, Kobuchizawa), who donated lectin extracts and Dr. David Cass, Pat Seymour (Devonian Gardens), and Rudy Kroon for obtaining plant seeds and roots. I would also like to thank Dr. Michael Longenecker, who generously donated the monoclonal antibodies used in the present study. Lastly, but not the least, I wish to thank Donna Taylor and Eva Dimitrov for their expert technical assistance.

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List of Abbreviations

#	serum number
α	anti e.g. L11=anti line 11
C1	first complement conponent
C	Control
C_1	cells in saline
C_2	cells incubated with antiserum
C_3	cells incubated with complement
FCS	fetal calf serum
HPBS	Hanks 0.75% phosphate buffered saline
L	line
ml	milliliter
μl	microliter
MBB	Modified barbital buffer
NS	normal serum
PBS	phosphate buffered saline
RBC	red blood cell, erythrocyte
RT	room temperature
PHA	phytohemagglutinin

I. Introduction

While chicken blood grouping has been studied extensively (Wong et al '72, Schierman & Nordskog '62, '65, Gilmour et al '76, Fredericksen et al '75, '77), there are only a few studies of blood groups in the Japanese quail (Mizutani et al '77a, b, c, '81c, Perramon et al '66, Perramon '69, '75, Katoh & Wakasugi '80, '81). Mizutani et al ('77a) tested the extracts of 84 plants for their ability to agglutinate erythrocytes (RBC) of five strains of quail. Some lectins agglutinated the RBC's of some individuals of either sex or the RBC's of some females whose reaction depended upon their laying status. Blood groups detected by the PHA's of peanut, soybean, and *Naematoloma sublateritium* were found to be under the control of simple autosomal genes (Mizutani et al '77b, c, & '81c).

Perramon ('75) described four quail blood group systems, A, B, C, & D, detected by hemagglutination with alloantibodies produced by immunization with whole quail blood. The A system includes antigens A^1, $A^{1'}$, A^2, $A^{3'}$, and A^3. The B system is represented by one antigen, B^1, which segregates with A^2. The C RBC blood group was found only once, and is yet to be reproduced, while the D group occurs frequently and appears complex. The latter group requires further investigation. All four blood group systems are thought to be autosomal and genetically independent although some linkage is seen through segregation.

Katoh & Wakasugi ('80) immunized quail, rabbit, and Holstein cows with quail RBC suspensions. The specificity of each antiserum for A, B, & C antigens, as detected by hemagglutination, was the same as that originally detected by natural antibodies. Katoh & Wakasugi ('81) immunized quail with quail lymphocytes and obtained reproducible antisera which defined lymphocyte antigens Ly_1 and Ly_2. Ly_1 and Ly_2 antigens were found on RBC's, leukocytes, and spleen cells and are thought to be controlled by co-dominant autosomal genes. The Ly_1 specifity showed a similarity to the A specificity.

To date, none of the quail blood group systems have been described in the context of their possible importance or their physiologic or immunologic significance. The published descriptions appear as isolated cases and confusion arises from the terminology used. The terms used by Perramon, and Katoh & Wakasugi, e.g. the A, B, & C blood group systems, may refer to very different antigen systems. These studies have concentrated on blood antigens located on the surfaces of RBC's and use hemagglutination assays. Furthermore, even though tested with numerous strains of quail, there have been no attempts to relate blood groups to histocompatibility comparable to what has been done for the B system of the chicken (Pazderka et al '75).

The present study compares inbred lines of quail which are internally histocompatible and mutually histoincompatible (Sawada et al, unpublished) by: 1)

hemagglutination with lectins, 2) RBC hemolysis with alloantisera produced by immunization with thymus and spleen cells, or whole blood, and 3) complement-mediated lymphocyte cytotoxicity with these alloantisera.

II. Materials and Methods

A. Experimental Animals

Inbred lines of *Coturnix coturnix japonica*, established at the University of Alberta by full-sib matings, were used in this study. Four highly inbred lines were found to be histocompatible within lines and histoincompatible between the lines by skin grafting experiments (Sawada et al). These lines were designated lines (L) 11, 27, 71, and 75. L 99 was used in a second set of immunizations and originates from a cross between L 11 and L 84 from which the L 11 homozygotes were recovered. Two partially inbred lines, L 68 and L 70, were also used in the lectin-RBC agglutination experiments. Birds used in this study from L 11 and L 27 were inbred to 12-13 generations while the remaining lines used were inbred to 5-7 generations.

B. Bleeding

One ml blood samples were obtained from the jugular veins of the quail in 1 ml syringes (no. 23g needle) using either 0.1 ml heparin (100 units Organon Porcine mucosa extracted heparin) or 0.1 ml 10% sodium citrate in Gibco 0.75% phosphate buffered saline (PBS) as the anticoagulent since both were found to be equally effective. Blood samples

were washed three times with PBS with the exception of the blood samples prepared for complement-mediated RBC hemolysis. It was found that RBC integrity could be maintained for longer periods when the RBC's were suspended in PBS rather than ordinary 0.75% saline. When necessary blood samples were stored in 0.02% sodium azide at 4°C for periods not exceeding one week.

C. Preparation of the Lectins

Commercially prepared lectins

Commercially prepared lyophilized lectins were weighed out and dissolved in PBS. The pH's of the lectin solutions varied from 6.5-6.8. When not in use, these lectins were stored according to the manufacturers' instructions.

Lectins prepared from plant extracts

(i)Lectin extracts obtained from M. Mizutani

Extracts from M. Mizutani[1] were divided into 1.0 ml aliquots and stored at -10°C. Prior to use, the extracts were thawed and centrifuged at 1000 rpm for 15 minutes to remove any precipitates formed. An IEC Centra-7R centrifuge was used for all centrifugations less than 4000 rpm and a

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[1]Nippon Institute for Biological Science, Kobuchizawa

Servall superspeed RC-2 was used for centrifugations at 4000 rpm. The extracts were subsequently diluted with PBS where necessary.

(ii)Lectins obtained from seed and root extractions

Crude seed and root extracts were prepared by slight modifications of Mizutani et al ('77c). Seeds or roots were soaked in 0.75% saline for 24-48 hours and then homogenized with an equal volume of PBS. The homogenate was filtered through Whatman no. 3 filter paper. Extracts were obtained by centrifuging the homogenate at 3000 rpm for 30 minutes, followed by heating at 60°C for 30 minutes, and once again centrifuged at 3000 rpm for 10 minutes. The supernatants were stored at -10°C.

D. Trypsin Treatment

Non-reactive lectins were tested again following treatment of RBC's with trypsin and neuraminidase. Trypsinized RBC's were prepared on the day of the agglutination test according to the method described by Lis and Sharon ('72). RBC's were collected from a blood sample by centrifugation at 2000 rpm for 5 minutes at room temperature in a clinical centrifuge and washed 3-4 times with 0.75% saline. The washed RBC's were suspended in PBS (to give a 10% RBC suspension). To 10 ml of the RBC suspension was added 1 ml of a 1% solution of trypsin

(Bovine pancreas, Type III, Sigma) 1:99 PBS, and the mixture was incubated for 1 hour at 37°C. The trypsinized RBC's were washed 4-5 times with 0.75% saline to remove the last traces of trypsin and were suspended in PBS to give the standard RBC suspension used in lectin-RBC agglutination tests (i.e. 0.5%).

E. Neuraminidase Treatment

Treatment of RBC's with neuraminidase (Clostridium perfringens, Type VI, Sigma) was carried out as described by Stratton et al ('73) by mixing 2 parts of a neuraminidase solution (2 units/ml dH_2O) with 10 parts of a 10% RBC suspension previously washed twice with PBS. After incubation for 1 hour at 37°C, the cells were washed with 4 changes of PBS and suspended in PBS to give a 0.5% RBC suspension.

F. Erythrocyte Agglutination Tests

Agglutination tests were performed in Cooke (Dynatech Labs) polystyrene 12.7 x 9.5 cm, 96 rounded-well microtiter plates. The tests were performed by twofold serial dilution of the lectin solutions: 100 microliters (ul) of a 0.5% quail RBC suspension were added to 100 ul of each of the diluted lectin solutions. The mixture was shaken manually, left for one half hour at room temperature and shaken again.

The mixture was incubated at room temperature for one hour after which the agglutination pattern was recorded. The plates were covered with plate covers and cellophane to prevent dehydration and were then incubated overnight at 4°C. The agglutination pattern was then recorded again. Controls consisted of blood samples in PBS.

G. Immunizations and Alloantisera Preparation

Alloantisera were prepared by intraperitoneal injection (IP) with 0.5 ml whole blood or spleen and thymus cell suspension. Thymus and spleen were removed and cut into small pieces with fine scissors. The tissues were passed through a nylon sieve and flushed with 1 ml Hanks (Gibco) 0.75% PBS (HPBS), washed in HPBS twice, and adjusted to 10^5-10^6 cells/ml. The immunizations were directed towards preparing line specific anti 11, anti 27, anti 71, and anti 75 antisera by doing the immunizations between lines (e.g. L 11 cells into L 71 recipients). Recipients were immunized once a week for a period of six months.

The first antisera were not collected until the birds had been immunized weekly for a period of two months. One ml blood samples were collected from the jugular vein with sodium citrate 2 days after the weekly booster. One batch of antisera was collected 3 days after the weekly booster immunization to determine if 48 hours was sufficient time to produce potent antiserum.

The blood samples were spun down at 1500 rpm for 10 minutes and 0.5mM calcium chloride was added to the supernatants to precipitate fibrin. The antisera were collected from the supernatants following centrifugation at 4000 rpm at 4°C for 20 minutes. Initially, four antisera were produced against L 11, 27, 71, and 75. The antisera from each line were pooled and stored at -70°C. In addition, normal or non-immune sera were collected from each line. For use antisera were thawed and any precipitates present were removed by centrifugation at 4000 rpm at 4°C for 20 minutes. The antisera were heated at 56°C for 20 minutes to inactivate any endogenous complement. Precipitates formed by this heating were also removed by centrifugation at 4000 rpm for 20 minutes. Only males were used for immunization as blood collected from males contains very little lipid as compared to that of females (Schram and Christenson '71). The level of circulating antibodies in laying females is less than that of males (Perramon '66) and in this study females were reserved for breeding.

H. Preparation of Monoclonal Antibodies

Mouse monoclonal antibodies to chicken lymphocytes and RBC B blood groups were generously provided by Dr. B.M. Longenecker.[2] Mouse anti-chicken lymphocyte monoclonal antibodies were of two sources, ascites supernatant or

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[2](University of Alberta, Edmonton)

culture medium supernatant. The ascites supernatants, obtained frozen, were thawed and diluted 1:500 in PBS + 5% FCS for use. Culture supernatants, obtained and stored at 4°C, were used undiluted. Mouse anti-chicken RBC B blood group monoclonal antibodies Ch 62 (14/14), Ch 45 (15/15), and Ch 32 (19/19) were diluted 1:100 in PBS + 5% FCS while Ch 5 (2/2) and LC-5 (21/21) were diluted 1:500 in PBS + 5% FCS. All remaining antibody solutions were stored frozen after use.

I. Complement-Mediated Erythrocyte Hemolysis

Complement-mediated hemolysis tests were performed similarly to that already described (Garvey et al '77). Antiserum (100 µl) diluted with modified barbital buffer (MBB) was added to glass test tubes (6 x 13 mm). An equal volume of MBB containing 1% RBC's, previously washed three times in HPBS, was added to the antiserum and mixed by inversion. The tubes were incubated at room temperature for 10 minutes and 100 µl of guinea pig complement (Flow Labs), diluted 1:4 with MBB, were added. The tubes were inverted and incubated at 37°C for 30 minutes and centrifuged at 1500 rpm for 10 minutes. The colour intensity was scored by comparison with controls. Controls included tubes containing the same mixture minus complement, minus antiserum, or minus both antiserum and complement.

J. Preparation of Quail Lymphocytes

The Ficoll-Hypaque method was used to isolate lymphocytes from whole blood (Lightbody '76, Amos & Pool '76). One ml of whole blood from non-immunized birds was spun down at 1500 rpm for 10 minutes. The buffy coat was mixed with an equal volume of RPMI 1640 medium (Gibco) containing glutamine and buffered to a pH of 7.4 with 0.20mM Hepes (Sigma). The mixture was carefully layered on an equal volume of Ficoll-Hypaque (Histopaque, Sigma) and centrifuged at 2000 rpm for 12 minutes. The interface containing lymphocytes was collected and washed three times in RPMI medium. It was found that cell viability could be increased by suspending the lymphocytes in media rather than in HPBS. After washing, the lymphocytes were resuspended in 1 ml of media. The number of viable cells was counted in a trypan blue or eosin solution by their ability to exclude these dyes (Terasaki & McClelland '64) using a hemocytometer, a Wild microscope, and a 40x objective, and then adjusted to 10^6 cells/ml with RPMI. Quail lymphocytes tested with monoclonal antibodies were obtained as described above, but were suspended in RPMI + 10% fresh fetal calf serum (FCS, Gibco).

K. Preparation of Chicken Lymphocytes

Chicken lymphocytes, isolated from blood samples (Ellerslie Farms, University of Alberta) as described for the isolation of quail lymphocytes, were suspended in RPMI + 5% FCS and adjusted to 10^6 cells/ml.

L. Complement-Mediated Lymphocyte Cytotoxicity

The lymphocyte cytotoxicity test was somewhat modified from the one previously described (Mittal et al '68, Gorer & Gorman '56). Microdroplet Terasaki plates (Falcon 3034) were prepared by filling the wells with 5 µl paraffin oil (Fischer Scientific Co.) to prevent evaporation. One µl of antiserum was added to the wells. One µl of the lymphocyte suspension was subsequently added to each well with care being taken not to touch the antiserum. The plates were manually shaken to ensure mixture of cells with antiserum. The plates were incubated at room temperature for 30 minutes. Five µl of reconstituted rabbit complement (Cedarline Labs) diluted 1:32 with ice-cold, distilled water were then added. The plates were shaken and incubated at room temperature for one hour. Two µl of aqueous eosin were then added. After 3-5 minutes 5 µl of 40% formaldehyde, adjusted to a pH of 6.0-7.0 with KOH, were added. A 50 x 75 mm coverslip was lowered onto the wells. Controls consisted of wells containing lymphocytes in RPMI, tests minus

complement, and tests minus antiserum.

Reactions were read with an inverted Wild phase contrast microscope using a 40x objective. Living lymphocytes were refractile whereas dead lymphocytes stained with eosin. A further indication of lymphocyte viability was cell size as indicated by other authors (Terasaki et al '60, Walford '60). Living lymphocytes were smaller than dead lymphocytes. A negative score was one in which the cell viability was the same as the controls. Maximal cell survival in controls was 95% and minimal cell survival was 75% (5-25% killed). A positive score was one in which the reaction was clearly different from the controls (25-100% killed). a weakly positive score was one in which the cell death was only slightly greater than controls (25-40% killed) and such scores were noted as negative scores. Representative negative and positive reactions were photographed with Ilford Pan F 50 ASA film at 400x magnification on a Zeiss photomicroscope III.

M. Characterization of the Alloantisera

To ensure that reading represented the effects of antibodies, lymphocyte cytotoxicity tests were run with antiserum in parallel with normal serum for each line.

The cross-reactivities of the antisera were examined by testing a two-fold serial dilutions of each antiserum with all lines.

RBC-specific and lymphocyte-specific alloantisera were prepared by absorption according to the method described by Katoh & Wakasugi ('81). RBC-specific alloantiserum was prepared for hemolysis by absorption with combinations of RBC suspensions from the histoincompatible lines. Lymphocyte-specific alloantiserum was prepared for cytotoxicity by absorption by one of two ways. RBC's and lymphocyte suspensions from each line were separated via Ficoll-Hypaque. Each antiserum was absorbed first with the combined RBC suspensions from the remaining histoincompatible lines and then the partly absorbed antiserum was likewise absorbed with the combined lymphocyte suspensions from the histoincompatible lines. Alternately, each alloantiserum was absorbed with whole blood suspensions from the histoincompatible lines. In each case the blood suspensions (RBC's, lymphocytes, or whole blood) were made into a 1% solution with HPBS. Three ml of each combination of blood suspensions were spun down at 1000 rpm for 3 minutes. The supernate was discarded as cleanly as possible. Two hundred ul of the appropriate antiserum, previously heated, were added to the blood mixture. The mixture was incubated at 4°C for one hour with several shakings. The absorbed alloantiserum was obtained by centrifugation at 1000 rpm for 15 minutes. In addition, alloantiserum was absorbed with isolated RBC's from within histocompatible lines.

N. Reproducibility of Alloantisera Preparation

A second group of birds, L 71 and L 99, was immunized according to the procedure outlined above for the preparation and collection of line-specific alloantiserum. The birds were immunized for ten weeks. The antisera were collected following eight weeks of immunizations and used in lymphocyte cytotoxicity tests. The sera were tested for potency against normal antisera and cross-reactivity with the remaining histoincompatible lines. Lymphocyte-specific alloantiserum was prepared by absorbing each antiserum with whole blood from the remaining histoincompatible lines.

O. Cytotoxicity Tests with Monoclonal Antibodies

Cytotoxicity tests with monoclonal antibodies were done in two ways. The first is that described above and the second is slightly modified from that described by Longenecker (personal communication). For simplicity, when referring to cytotoxicity tests with monoclonal antibodies, the test described by the author will be referred to as the room temperature (RT) cytotoxicity test while the modified test according to Longenecker will be referred to as the heated (37°C) cytotoxicity test. The test conditions described by Longenecker were maintained. The volumes were reduced, but the ratios of volumns maintained so that tests could take place in Terasaki microtiter plates. Five µl

paraffin oil were added to each well and 3 µl monoclonal antibodies (diluted as described above) were added. One µl lymphocytes was added and the cells and antibodies were incubated at room temperature (RT) for 1 hour after which 3 µl rabbit complement diluted 1:16 with PBS + 5% FCS were added. The mixture was incubated at 37°C for 30 minutes. Two µl eosin were added and after 2-3 minutes 5 µl formaldehyde were added to fix the cells. Reactions were scored visually as described earlier on a Wild phase microscope with a 10x or a 40x objective. Controls were cells + complement, cells + antiserum, and reaction conditions with chicken lymphocytes. Quail and chicken lymphocytes were tested with mouse anti-chicken RBC B blood group monoclonal antibodies in both the RT and the 37°C cytotoxicity tests while quail and chicken lymphocytes were tested with mouse anti-chicken T-cell monoclonal antibodies in the RT test only.

Quail and chickens used in tests with monoclonal antibodies are identified in Appendix 5.

III. Results

A. Lectin-RBC agglutination

Twenty five lectins were tested for their ability to differentiate between quail RBC's. The lectins examined can be grouped into two categories (Table 1). Certain lectins agglutinated all quail RBC's regardless of line or sex and the remaining lectins failed to agglutinate any quail RBC's regardless of line or sex. Differential RBC agglutination was not evident among females. The non-reactivity by lectins, which previously did not agglutinate all quail RBC's, did not change when tested with trypsinized and neuraminidase treated RBC's. It was not possible to confirm differential agglutination of RBC's by lectins as reported by others (Mizutani et al '77a,b,c, & '81c).

B. Alloantiserum production

In the present study alloantisera were easily obtained. Because of the small quantities of antiserum obtained from individuals, the antisera were pooled. L 71 was chosen as the alloantisera producing line for L 11, 27, & 75 and L 75 as the alloantiserum producing line for L 71. Antisera collected 2 or 3 days following weekly immunization were equally potent. All birds used in complement-mediated

hemolysis and lymphocyte cytotoxicity are identified in Appendix 2 and all antisera are listed and characterized in Appendix 3. The antisera are numbered to correspond to the way they appear in Tables 2 through 12.

C. Complement-mediated RBC hemolysis

Line discrimination was seen in RBC hemolysis tests. Complement-mediated hemolysis titers were highest for tests with antiserum produced against that line and RBC's from that line (Table 2). Individual 40 was tested on two separate occasions and gave the same titer illustrating the reproducibility of the test. In all cases normal (non-immune) sera failed to react. Three controls were conducted consisting of: cells in MBB (C_1), cells incubated with antiserum (C_2), and cells incubated with guinea pig complement at 1:10 (C_3). All controls were negative.

All cross-reactivity was lost following absorption of each antiserum with RBC's of the lines other than the one used for immunization (Table 3). What remained was line specific RBC activity with titers that were less than those seen in Table 2. Controls were the same as those described above and all were negative.

D. In Vitro Quail Lymphocyte Survival

Quail lymphocyte survival 'in vitro' was precarious. Survival depended greatly on the concentration of FCS in the medium. Quail cells suspended in RPMI or RPMI + 5% FCS remained viable (up to 95% viable) when maintained at 4°C or room temperature. Such cells died if heated at 37°C. Survival after heating at 37°C could be increased to 60-70% when the cells were suspended in RPMI + 10% (or more) FCS, but concentrations of FCS greater than 5% caused cloudiness that obscured visual scoring of the cells.

E. Complement-mediated lymphocyte cytotoxicity

In preliminary complement-mediated lymphocyte cytotoxicity tests it was found that whenever undiluted, reconstituted rabbit complement was present all cells were destroyed. Cells burst and only cellular debris and fragments were visible. Rabbit complement was toxic to quail lymphocytes. This was prevented by diluting the reconstituted complement with distilled, ice cold water. Table 4 illustrates a gradation of rabbit complement dilutions prior to incubation with lymphocytes to determine an endpoint where cell destruction did not occur. Following a dilution of complement to 1:16, cell destruction did not occur in 12 of 13 tests. Individuals 14 and 41 gave the same endpoint of loss of complement toxicity when tested a second time. A

1:32 dilution of rabbit complement was chosen for use in all future lymphocyte cytotoxicity tests.

A series of cytotoxicity tests with alloantisera was performed over a range of complement dilutions (Table 5). Tests were positive with complement dilutions greater than 1:16. However, 6 of 15 cases showed some cell destruction at complement dilutions 1:16 and 1:32. Individual 14 gave the same results when tested a second time. The range of complement dilutions in which cytotoxicity tests were positive (Table 5) matches the range of non-toxic complement dilutions (Table 4).

Figures 1 to 3 illustrate lymphocyte cytotoxicity tests. Figure 1 shows a positive scoring. A negative scoring is shown in Figure 2. Positive, stained, and negative, unstained, cells are shown together in Figure 3.

Complement-mediated lymphocyte cytotoxicity tests showed: line specificity, a moderate degree of cross-reactivity, unreactivity of normal serum, and negative controls (Table 6). A striking feature of this assay is its greater sensitivity as compared to the complement-mediated hemolysis test (Table 2). Titers in cytotoxicity tests were 7-9 tubes greater. Anti-71 antisera did not show any cross-reactivity and anti-75 antisera showed cross-reactivity from L 11 and 27 only. Individuals 8, 21, and 41 gave the same or similar titers when tested twice with their respective antisera. Controls were: cells in RPMI (C_1), cells incubated with antiserum (C_2), and cells

incubated with diluted rabbit complement 1:32 (C_3), and all were negative.

Lymphocyte cytotoxicity was strongly line specific after the absorption with RBC's from histoincompatible lines (Table 7) yet at titers lower than for unabsorbed antiserum (Table 6) and some cross-reactivity remained. Cross-reactivity was lost with further absorption of the partly absorbed antisera with lymphocytes from the histoincompatible lines (Table 8). Line-specific activity remained, but at yet further reduced titers. Absorption of antiserum with whole blood from histoincompatible lines was performed (Table 9). Similarly, the two-step absorption (Tables 7 & 8) with whole blood from the remaining histoincompatible lines reduced cross-reactivity. L 11 and L 99 showed reciprocal cross-reactivity to the same extent after absorption.

It appears that there exist several quail blood antigens. These can be grouped into: (1) common quail antigens present on all RBC's and lymphocytes (Table 6), (2) common quail RBC antigens (Tables 2,10), (3) line-specific quail RBC antigens (Table 3), (4) common quail lymphocyte antigens (Table 7), (5) line-specific quail lymphocyte antigens (Table 8), and (6) shared line-specific quail RBC and lymphocyte antigens (Table 10).

Effect of Age on Cytotoxicity

The effect of age was examined on quail alloantiserum production and lymphocyte reactivity in complement-mediated cytotoxicity (Table 11). While lymphocytes of young and old quail reacted equally well in alloantiserum cytotoxicity tests, the production of potent alloantiserum decreased with increasing age as shown by an individual in Table 11.

Naturally Occuring Alloantibodies

A search was made for naturally occurring alloantibodies like those reported by Katoh & Wakasugi ('80) between the lines of quail (Table 12). All lines were subjected to complement-mediated lymphocyte cytotoxicity tests with normal serum from each of the five quail lines. No naturally occurring alloantibodies were found.

F. Cytotoxicity Tests with Monoclonal Antibodies

Mouse anti-chicken-lymphocyte monoclonal antibodies failed to react with all quail cells tested (Table 13). Controls consisting of quail cells plus complement, cells plus antiserum, chicken lymphocytes plus complement, and chicken cells plus antiserum were also negative. Chicken lymphocytes were positive with antisera from ascites supernatants (24C8, 24C30, 24C31, and some 24C35) and culture medium supernatants (25C23, 26C1, 26C10, 26C22, 26C23, 26C27, 26C34, 26C35, 26C36, 26C43, and 26C45).

Mouse anti-chicken RBC B blood group monoclonal antibodies failed to react with all quail and chicken lymphocytes tested by both the RT cytotoxicity test and the 37°C test (Tables 14 & 15). All controls were negative.

IV. Discussion

The present study examined a family of histocompatible lines of Japanese quail in an attempt to detect line-specific molecular markers on the surfaces of erythrocytes or lymphocytes. Such markers could provide an efficient method of selecting and matching histocompatible quail and might contribute interpretatively useful information.

Briles et al ('50) showed the existence of inherited RBC molecular markers defining the B system, which was later shown to be representative of the Major Histocompatibility Complex (MHC) of the chicken. One would expect to find co-inherited molecular markers associated with histocompatibility in quail.

Lectin-sugar interactions can be likened to antibody-antigen interactions by their specificity for sugars on cell surfaces and the presence of two binding sites as has been seen for Con A and soybean lectin. They are unlike antibody-antigen interactions in that their binding sites are homogeneous and identical. Studies of lectin-sugar reactions can serve as a model for and provide information relevant to antibody-antigen reactions (Sharon & Lis '72). Lectins, or phytohemagglutinins, are proteins that have the ability to agglutinate cells, particularly RBC's. Their availibility, diversity, and sugar specificity make them excellent reagents for blood typing (Boyd '63).

Human blood groups A, B, O, H, & Le (Boyd '63, Bird '59, Boyd & Reguera '49, Ottensooser & Sato '63) were detected by hemagglutination with lectins. Blood typing with lectins has been extended to animals such as mice and birds. Mizutani et al ('81b) tested three strains of mice (BALB/c-hr, dd, and wild) with 111 phytohemagglutinins. Although he was unable to detect strain differences, individual differences were found. Lectin from the peanut (*A. hypogaea*) which agglutinated some mouse RBC's, but did not hemolyze all mouse RBC's and hemolyzed some mouse RBC's, was used in the present study. Mizutani et al ('80) tested the extracts of 97 plants to study the agglutinability of pigeon RBC's with PHA's. Of these tested, 13 agglutinated the RBC's of some males and some females. *Sophora japonica* and *Pisum sativum* (Mizutani et al '81) agglutinated some female RBC's, males were negative. *Discorea batatus* and *Osmunda japonica* agglutinated some male and some female RBC's. *P. sativum*, *Vicia fava*, and *Lathyrus odoratus* agglutinated the RBC's of laying female chickens (Fujio & Mizutani '75), and *P. sativum* was also found to differentially agglutinate individual mouse, rat, guinea pig, and rabbit RBC's (Mizutani & Fujio '71). Miller ('65) reported differential agglutination with *A. hypogaea* and *Bandieraea simplicifolia* with RBC's of ring-necked doves. Scheinberg & Reckel ('61a,b) reported differential agglutination of chicken RBC's by *Pisum arvense*, and identified the 'Hi' agglutinogen in chickens. Some laying

females were positive, and all males tested were negative. The Hi agglutinogen is under the control of an autosomal gene plus estrogens. The expression of the Hi antigen can be induced in some males and immature chickens by injections of estrogens (Schienberg & Reckel '62).

Lectins were used to differentiate Japanese quail RBC's by Mizutani et al ('77a). Of 84 lectins, eight gave differential hemagglutination: *Abermoschus esculentus*, *Discorea batatus*, *Pteridium aquilinum*, *Osmunda japonica*, *Arachis hypogaea* (peanut), and *Glycine max* (soybean). The soybean lectin agglutinated the RBC's of some laying females (Mizutani et al '77b). Individual differences detected by peanut lectin were also found in chickens, turkeys, and ducks, (Mizutani et al '77c). *A. hypogaea* agglutinated some male and some female chicken and turkey RBC's, and some female duck RBC's. Drake RBC's were not agglutinated by this peanut lectin.

A variety of commercially prepared and crude lectin extracts were tested for their ability to differentially agglutinate quail RBC's. All lectins tested, including those obtained directly from M. Mizutani, failed to discriminate between quail lines and individuals. Differences were not detected between males and females or among females. Trypsin and neuraminidase treatment of the RBC's did not uncover reactive sites for lectins. It was not possible to confirm Mizutani's results. This may be due to a difference between the quail Mizutani used, as well as Perramon's (see below),

and those used in the present study. Both Mizutani and Perramon's quail consist of various strains, and unlike those used here, are not established, highly inbred, and histocompatible lines. It appears that lectins do not detect the line-specific antigens of histocompatibility or other gene complexes in our lines of Japanese quail.

The Japanese quail has been shown to be a good avian model for immunological studies (Perramon '69, '75). Perramon ('69) demonstrated the advantages and ease with with quail could be used to produce alloantibodies. Its advantages include: ease of handling, short generation time, ability to withstand long, rigorous, and variable immunization periods and frequent bleedings, and the small volume of blood required for potent antibody production. Perramon ('65) produced quail alloantiserum following immunization with RBC suspensions which detected RBC systems A, B, C, & D by hemagglutination. Data (unpublished) from hemagglutination tests on the University of Alberta quail using Perramon's alloantisera specific for RBC antigens A', $A^{1'}$, A^2, A^3, $A^{3'}$, and B' indicate that these antigens are detectable on RBC surfaces. Some L 11 RBC's were agglutinated by A', A^2, and $A^{3'}$ while no L 11 RBC's were agglutinated by $A^{1'}$ and A^3. Individual agglutination differences were seen among L 71 birds by A', $A^{1'}$, A^2, and A^3. All L 71 individuals were agglutinated by $A^{3'}$. Erythrocytes of a single L 27 individual were agglutinated by A^2 and $A^{3'}$. All other L 27 individuals and L 75

individuals failed to react with all alloantisera. All four lines failed to react with B' antiserum. There did not appear to be segregation of B' with A^2. Individual differences were seen, not line differences, suggesting that these RBC antigens are not related to histocompatibility or line differences.

Avian RBC typing has been performed by hemagglutination on the assumption that avian antibodies cannot bind mammalian complement, particularly mammalian C1 (Stolfi et al '71, Okazaki et al '62, & Benson et al '61) so that C1 cannot activate and fix the remaining complement components (Muller-Eberhard '75). On this basis complement-fixation, complement-mediated hemolysis, and complement-mediated lymphocyte cytotoxicity tests have been used only infrequently for avian systems. Reports of the inability of avian antibodies to bind mammalian complement come from work on chickens (Stolfi et al '71, Rice '47, Okazaki et al '62, Jankovic & Isakovic '60), turkeys (Okazaki et al '62, Benson et al '61, Rice '47, '48, Rose & Orlans '62a,b), and ducks (Rice '47,'48). The addition of purified avian C1 (Benson et al '61) or normal avian serum (Brumfield et al '61, Rose & Orlans '62a,b, Orlans et al '62a,b) is required for the remaining mammalian complement components to become activated. Yet, there are reports of the fixation of guinea pig complement by the antibodies of sea-shore birds (Pollard '42), parrots (Meyer et al '39), sparrows, Adelaide rosella, red rump parrots (Beech & Miles '53), and pigeons (Eddie et

al '42, Lazoffsky '47, Meyer et al '42, Karrer et al '50, Beech & Miles '53, Terzin '60).

Alloantiserum produced against each line of quail was reactive in guinea pig complement-mediated hemolysis tests. Unlike the lectin and alloantisera (Perramon's antisera) hemagglutination tests, hemolysis tests showed line differences. Each antiserum reacted most strongly with its respective line. However, cross-reactivity with other lines was seen, and some cross-reactivity remained after absorption. Contrary to the reports that avian antibodies cannot bind mammalian C1, the data seen here show that quail antibodies bind guinea pig complement. Interchangability of complement components was shown by Cushing ('50a,b) by the interaction between frog, carp, and guinea pig complement components. There may be significant species specificity. Quail, pigeon, and dove antibodies can bind mammalian complement, but chicken, turkey, and duck antibodies may not. The binding of mammalian complement by quail antibodies demonstrated in the present study may be the result of an intense search for the right conditions.

Since alloantiserum and lectin hemagglutination show no association with histoincompatibility, and hemolysis shows very weak association, lymphocytes were examined in search of strong reactions associated with or co-inherited with histocompatibility differences. Previous lymphocyte typing was done by lymphocyte agglutination for the same reason as erythrocyte typing, the impression that avian antibodies do

not bind mammalian C1. Schierman & Nordskog ('62) detected chicken A, D, & L, erythrocyte antigens on lymphocytes by agglutination, but were unable to detect antigens belonging solely to the lymphocytes. B, C_3, and C_4 antigens were also found on lymphocytes by lymphocyte agglutination (Schierman & Nordskog '65). Frederickson et al ('75, '77) described the chicken T-cell antigen Ly-4 by inhibition of GVH splenomegaly and lymphocyte agglutination.

The first report of avian lymphocyte cytotoxicity testing for blood typing was reported by Gilmour et al ('76). They detected B-cell antigen Bu-1 and T-cell antigen Th-1 and showed that they are not related to the B blood group system. In Gilmour's cytotoxicity test isolated chicken C1 was added to guinea pig complement devoid of C1 (i.e. C2-9) in support of the theory of the inability of avian antibodies to bind guinea pig complement (C1). The lymphocytes were incubated with antiserum and C1 in a water bath at 30°C for 20 minutes. Guinea pig C2-9 was added to the mixture and incubated at 37°C for 45 minutes. The mixture was removed from the bath and trypan blue added. The cytotoxicity test used here differs from Gilmour's in that it was run at room temperature because of the poor 'in vitro' survival of quail lymphocytes at higher temperatures. Complete diluted rabbit complement was effective with quail alloantisera and it was not necessary to add a source of avian C1. Lymphocyte cytotoxicity tests were strongly line-specific at titers several fold greater than hemolysis

titers. Titers declined with the age of the antiserum producer. Absorption with lymphocytes of different lines removed line cross-reactivity and the final reactions were line-specific.

Having previously described the origin and relation of L 99 to L 11 (see Experimental Animals, Materials and Methods), it would appear that the alloantiserum is selecting for the MHC (major histocompatibility complex) because the reciprocal cross-reactions between L 11 and L 99 are much the same following absorption (Table 9). Absorption may remove common population antigens and L 84 antigens leaving those antigens determined by the 11 gene. It would be necessary to isolate and characterize the molecular markers or antigens detected by these alloantisera as has been done by Ziegler and Pink ('75) who isolated and characterized the chicken's B antigens by indirect immune precipitation, in order to find out if these markers represent MHC differences. Normal quail serum did not react in any tests of RBC's or lymphocytes.

This test, though reproducible, accurate, economic, and sensitive, is time-consuming. Approximately 5 hours are required to complete a test if one includes the collection of the blood samples, isolation of the lymphocytes, preparation of the antiserum, and completion of the test. An additional one half hour is required to score each plate made (usually 6-8 plates are made per test). Isolation of the lymphocytes requires approximately 45 minutes by

Ficoll-hypaque. The reaction of the lymphocytes, alloantiserum, and complement require approximately two and one half hours of which one and one half hours is incubation time (one half hour for lymphocytes with antiserum followed by 1 hour incubation after the addition of complement). Since the present test is based on that described by Mittal et al ('68), it seems reasonable that the test used here can be modified to fit the rapid 45 minute cytotoxicity test described by Mittal et al ('69). The cytotoxicity test (Terasaki et al '67) was shortened to the rapid cytotoxicty test by reducing the time required to isolate lymphocytes, reducing the incubation periods, and accelerating the scoring time (see Appendix 4).

Having produced line-lymphocyte specific alloantiserum, one would expect that the next step in characterizing line differences would be the production of monoclonal antibodies against each line. The close relationship of the quail and the chicken, both belonging to the subfamily Phasianidae (Haley et al '76), suggested that existing monoclonal antibodies to chicken antigens might react with quail cells. In a preliminary set of experiments, quail lymphocytes were tested with mouse monoclonal antibodies to chicken T-cell Marek's lymphoma and RBC B blood groups 2/2, 14/14, 15/15, 19/19, and 21/21.

None of the mouse anti-chicken lymphocyte monoclonal antibodies lysed quail cells, which suggests that the antigens detected by these antibodies are not present on the

quail lymphocytes. The failure of some of the antisera to lyse chicken cells suggests that these antisera may have denatured following storage at 4°C rather than having been frozen, or do not bind complement. The unreactivity of anti-chicken B blood group monoclonal antibodies may reflect the absence of B blood group markers 2/2, 14/14, 15/15, 19/19, and 21/21 on quail lymphocytes or these markers may be present, but undetectable in the preliminary tests. Some chicken's lymphocytes did not react in cytotoxicity tests, but did in agglutination and rosette tests (Longenecker et al '79, Mosmann et al '80). Mosmann et al '80 showed that by varying the type and conditions of an assay, it was possible to alter the specificity of monoclonal antibodies. Alteration of the test conditions or the test itself used in the present study may be required to show the presence of these antigens on quail lymphocytes if they exist. In the case of the chicken cells, the expression of these antigens is, however, restricted to RBC's (Longenecker and Mosmann '80).

It is noteworthy that the 'in vitro' survival of quail lymphocytes when heated at 37°C is greatly improved when the cells are maintained in the presence of larger than normal (10% vs 5%) amounts of fresh FCS. After heating at 37°C, lymphocyte survival is less than lymphocyte survival at room temperature (90-95% survival at RT and 60-70% survival at 37°C), but substantially greater than heated lymphocytes maintained in no or low (5%) amounts of FCS (0% survival).

V. Conclusion

In summary, having established histoincompatibile lines of Japanese quail by skin grafting experiments, investigations were made to detect line differences at the level of the quail erythrocytes and lymphocytes. Lectin and alloantisera hemagglutination tests failed to show an association of RBC molecular markers with histoincompatibility. Hemolysis tests detected weak differences between the lines. The low titers obtained and the high degree of cross-reactivity make this test undesirable for histoincompatibility typing. Lymphocyte cytotoxicity tests with rabbit complement detected strong line differences. Presumably, the alloantisera detect molecular markers or antigens present on the surfaces of the lymphocytes. This technique, though qualitative and time-consuming in terms of setting up the test conditions and reagents, and the time taken to do the test, is reproducible, accurate, economic, and highly sensitive. Lymphocyte cytotoxicity testing is a valid method for detecting line differences in this system of highly inbred, histoincompatibile lines of quail. It would be interesting to see if these findings are consistent throughout each line at generations other than at the level used in the present study.

Table 1

Lectin-Erythrocyte Agglutination

Lectin	Group no.
*Abermoschus esculentus	1
+Arachis hypogaea (peanut)	1
+Bandieraea simplicifolia	2
+Con A	1
+Con A IV	1
*Discorea batatus	2
+Dolichos biflorus (horse gram)	1
+Glycine max VI (soybean)	1
+Glycine max VII (soybean)	1
*Helianthus tuberosus	1
+Lens culinaris (lentil)	1
*Lepista nuda	1
°Mung bean	2
*Naematoloma sublateritium	1
*Osmunda japonica	1
+Phaseolus vulgaris (red kidney bean)	1
+Phytolacca americana (pokeweed)	2
°Pisum arvense (field pea)	1
+Pisum sativum (garden pea)	1
*Pteridium aquilinum	1
°Pteridium aquilinum	1
+Sophora japonica	2
+Tetragonolotus purpureas (asparagus pea)	2
+Triticum vulgaris (wheat germ)	1
+Ulex europeus (gorse)	2

where:

1 refers to lectins that agglutinate all quail RBC
2 refers to lectins that do not agglutinate any quail RBC
+ refers to commercially prepared lectins
* refers to lectins obtained from M. Mizutani
° refers to lectins prepared from extracts by the author

Table 2

Complement-Mediated RBC Hemolysis

Line	Bird no.	Titer: Antiserum Specificity & # NS	∝L11 (#1)	∝L27 (#2X)	∝L71 (#3)	∝L75 (#4)
11	15	N(#6)	4	-	-	-
	12	N(#5)	4	-	-	-
	3	N(#5)	4	-	-	-
	4	N(#5)	4	-	-	-
	7	-	4	3	3	3
	16	-	3-4	3	3	3
27	25	N(#12)	-	4	-	-
	20	N(#12)	-	5	-	-
	19	N(#12)	-	5	-	-
	32	-	1	5	2	3
	34	-	1	5	2	3
71	40	N(#20)	-	-	4-5	-
	40	N(#20)	-	-	4-5	-
	45	N(#21)	-	-	5	-
	41	-	N	N	5	3
	46	-	N	N	5	3
75	72	N(#28)	-	-	-	5
	84	N(#29)	-	-	-	5
	74	N(#29)	-	-	-	5
	86	-	1	2	2	5
	88	-	1	2	2	5

where:

N indicates a negative reaction
« refers to antiserum specificity e.g. L11 = anti-line 11 antiserum
refers to the number of the antiserum used and is described in Appendix 3
Controls consist of lymphocytes in RPMI, lymphocytes with antiserum only, and lymphocytes with guinea pig complement, and all were negative
Bird no. refers to the number of the bird identified in Appendix 2
NS refers to the normal serum used and is described in Appendix 3
Titers are the last positive antiserum serial dilution and are expressed so that a titer of 1 refers to undiluted antiserum, 2 refers to antiserum diluted 2^1, and 3 refers to antiserum diluted 2^2 prior to addition into the wells
- refers to those conditions where individual birds were not tested with the respective antiserum
Line refers to quail line

Table 3

Complement-Mediated RBC Hemolysis

following absorption with RBC from histoincompatible lines

Titer

Antiserum Specificity & #

Line	Bird no.	∝L11 (# 7)	∝L27 (#15)	∝L71 (#23)	∝L75 (#31)
11	16	1	N	N	N
27	34	N	2	N	N
71	37	N	N	2	N
75	80	N	N	N	3

where:

Controls were all negative and are as described in Table 2
Titers are the last positive antiserum serial dilution as described in Table 2

Table 4

Avoidance of Spontaneous Lymphocyte Destruction

with Dilution of Complement and Absence of Antiserum

		Titer							
Line	Bird no.	1	2	3	4	5	6	7	8
11	14	D	D	D	N	N	N	N	N
	14	D	D	D	N	N	N	N	N
	9	D	D	D	D	N	N	N	N
27	24	D	D	D	N	N	N	N	N
	34	D	D	D	D	N	N	N	N
	32	D	D	D	N	N	N	N	N
71	41	D	D	D	N	N	N	N	N
	41	D	D	D	N	N	N	N	N
	36	D	D	D	N	N	N	N	N
	39	D	D	D	D	N	N	N	N
75	88	D	D	D	D	D	N	N	N
	73	D	D	D	D	N	N	N	N
	84	D	D	D	D	N	N	N	N

where:

D refers to cells destroyed
N refers to cells remaining intact and unstained
Titer refers to the serial dilution of rabbit complement prior to addition into the well and a titer of 1 refers to undiluted complement, 2 refers to complement diluted 2^1, 3 refers to complement diluted 2^2, and 4 refers to complement diluted 2^3 respectively

Table 5

Determination of Lymphocyte Cytotoxicity

with Dilution of Complement and Antiserum Present

Line	Bird no.	^S	Titer 1	2	3	4	5	6	7	8
11	14	$L11^1$	D	D	D	+	+	+	+	+
	14		D	D	D	+	+	+	+	+
	9		D	D	D	+	+	+	+	+
	15		+	+	+	+	+	+	+	+
27	24	$L27^3$	D	D	+	+	+	+	+	+
	21		D	D	D	+	+	+	+	+
	32		D	D	D	+	+	+	+	+
	34		D	D	D	+	+	+	+	+
71	41	$L71^5$	D	D	D	D	+	+	+	+
	41		D	D	D	+	+	+	+	+
	39		D	D	D	D	+	+	+	+
	36		D	D	+	+	+	+	+	+
75	84	$L75^4$	D	D	D	D	+	+	+	+
	73		D	D	+	+	+	+	+	+
	88		D	D	D	D	+	+	+	+

where:

D refers to cells destroyed
+ refers to positive reactions
^S refers to antiserum specificity
Superscripts refer to the antiserum number (i.e. #)
Titer refers to the serial dilution of rabbit complement prior to addition into the well and is described in Table 4

Table 6

Complement-Mediated Lymphocyte Cytotoxicity

Line	Bird no.	NS	Titer: Antiserum Specificity & # ∝L11 (#1)	∝L27 (#2X)	∝L71 (#2Y)	∝L71 (#3)	∝L71 (#43)	∝L75 (#4)	∝L99 (#42)
11	14	-	13	-	-	-	-	-	-
	9	-	13	-	-	-	-	-	-
	8	N(#5)	13	-	-	-	-	-	-
	8	-	13	6	-	7	-	7	-
	13	-	13	6	-	7	-	7	-
	15	N(#6)	14	-	8	-	-	-	-
	5	-	-	-	-	-	4	-	7
27	24	-	-	11	-	-	-	-	-
	32	N(#13)	-	12	-	-	-	-	-
	33	N(#14)	-	12	-	-	-	-	-
	21	-	-	11	-	-	-	-	-
	21	-	5	13	-	6	-	5	-
	19	-	7	12	-	7	-	5	-
	30	-	-	-	-	-	1	-	2
	20	-	-	-	8	-	-	-	-
71	39	-	-	-	-	12	-	-	-
	36	N(#22)	-	-	-	12	-	-	-
	46	N(#21)	-	-	-	12	-	-	-
	41	-	-	-	-	12	-	-	-
	41	-	N	N	-	12	13	N	N
	45	-	N	N	-	13	-	N	N
	38	-	-	-	1	-	-	-	-
75	84	-	-	-	-	-	-	14	-
	73	N(#30)	-	-	-	-	-	13	-
	72	N(#28)	-	-	-	-	-	14	-
	87	-	5	3	-	N	-	14	-
	88	-	5	3	-	N	4	13	2

	80	-	-	-	3	-	-	-	-
99	96	-	-	-	-	-	2	-	12
	91	-	-	-	3	-	-	-	-

where:

Controls consisted of lymphocytes in RPMI, lymphocytes with antiserum only, lymphocytes with rabbit complement only, and all were negative
Titers are the last positive serial dilution of antiserum as described in Table 2

Table 7

Effects of Absorption on Lymphocyte Cytotoxicity:

A. absorption with RBC of histoincompatible lines

Line	Bird no.	C	Titer: Antiserum Specificity & # ∝L11 (#8)	∝L27 (#16)	∝L71 (#24)	∝L75 (#32)
11	2	N	8	1	1	2
	12	N	8	1	N	2
27	17	N	1	8	5	3
	19	N	1	8	5	3
71	44	N	N	N	6	N
	39	N	N	N	6	N
75	87	N	N	5	N	5
	80	N	N	5	N	5

where:
Control (C) consists of lymphocytes in RPMI
Titers are the last positive dilutions of antiserum as described in Table 2
Antiserum #8 absorbed with RBC's from L27, 71, & 75
Antiserum #16 absorbed with RBC's from L27, 71, & 75
Antiserum #24 absorbed with RBC's from L11, 27 & 75
Antiserum #32 absorbed with RBC's from L11, 27, & 71

Table 8

Effects of Absorption on Lymphocyte Cytotoxicity:

B. absorption with RBC followed by absorption with

lymphocytes of histoincompatible lines

		Titer				
			Antiserum Specificity & #			
Line	Bird no.	Control	∝L11 (#9)	∝L27 (#17)	∝L71 (#25)	∝L75 (#33)
11	2	N	5	N	N	N
	12	N	5	N	N	N
27	17	N	N	5	N	1
	19	N	N	5	N	N
71	44	N	N	N	3	N
	39	N	N	N	3	N
75	87	N	N	N	N	3
	80	N	N	N	N	3

where:
Control consists of lymphocytes in RPMI
Titers are the last positive dilutions of antiserum as described in Table 2
Antiserum #9 = #8 absorbed with lymphocytes from L27, 71, & 75
Antiserum #17 = #16 absorbed with lymphocytes from L11, 71, &75
Antiserum #25 = #24 absorbed with lymphocytes from L11, 27, & 75

Antiserum #33 = #32 absorbed with lymphocytes from L11, 27, & 71

Table 9

Effects of Absorption on Lymphocyte Cytotoxicity:

C. absorption with whole blood of histoincompatible lines

Titer

Antiserum Specificity & #

Line	Bird	αL11	αL27		αL71				αL75	αL99		
	no.	(#10)	(#18)	(#36)	(#26)	(#47)	(#48)	(#49)	(#34)	(#44)	(#45)	(#46)
11	2	-	-	-	-	-	2	-	-	-	3	-
	13	6	N	-	N	-	-	-	N	-	-	-
	15	-	-	3	-	1	-	-	-	4	-	-
	16	-	-	-	-	-	-	1	-	-	-	3
27	20	-	-	6	-	2	-	-	-	N	-	-
	22	N	5	-	N	-	-	-	N	-	-	-
	32	-	-	-	-	-	1	-	-	-	2	-
	34	-	-	-	-	-	-	N	-	-	-	N
71	40	N	N	-	5	-	5	-	N	-	N	-
	38	-	-	N	-	6	-	-	-	1	-	-
	44	-	-	-	-	-	4	-	-	-	-	1
75	87	-	-	-	-	-	1	-	-	-	-	2
	80	-	-	1	2	-	-	-	-	1	-	-
	83	N	N	-	N	-	-	4	-	-	-	-
	78	-	-	-	-	-	2	-	-	-	2	-
99	91	-	-	3	-	N	-	-	-	6	-	-
	89	-	-	-	-	-	N	-	-	-	5	-
	99	-	-	-	-	-	-	N	-	-	-	4

where:
Controls consisted of lymphocytes in RPMI and were negative
Titers are the last positive serial dilution of antiserum as described in Table 2

Table 10

Effects of Absorption on Lymphocyte Cytotoxicity:

D. absorption with RBC of histocompatible lines

Line	Bird no.	Titer: Control	Antiserum Specificity & #: ∝L11 (#11)	∝L27 (#19)	∝L71 (#27)	∝L75 (#35)
11	16	N	5	2	N	N
27	28	N	N	7	3	3
71	36	N	N	N	6	N
75	88	N	N	3	N	6

where:
Control consists of lymphocytes in RPMI and titers are expressed as the last positive serial dilution of antiserum as in Table 2
Antiserum #11 absorbed with RBC's from L 11 only
Antiserum #19 absorbed with RBC's from L 27 only
Antiserum #27 absorbed with RBC's from L 71 only
Antiserum #35 absorbed with RBC's from L 75 only

Table 11

Effects of Age on Alloantisera Production

and Lymphocyte Cytotoxicity

Antiserum	Age (mos) Antiserum Collection	Age (mos) Lymphocyte Cytotoxicity	Titer
71 anti 11[1]	7-9	7-9	12
71 anti 75[4]	7-9	7-9	11
71 anti 99[4,3]	6	6	11
75 anti 71[3]	7-9	7-9	12
99 anti 71[4,3]	6	15	11
71 anti 27[2]	2-9	7-9	13
71 anti 27[2]	15	15	7

where:

Titer refers to the last positive serial dilution of the antiserum prior to addition into the wells as described in Table 2
Superscripts refer to antiserum number (i.e. #)

Table 12

Tests for Naturally Occuring Alloantibodies

		Titer				
		Antiserum Specificity & #				
Line	Bird no.	NS L11 (#37)	NS L27 (#38)	NS L71 (#39)	NS L75 (#40)	NS L99 (#41)
11	5	N	N	N	N	N
27	30	N	N	N	N	N
71	41	N	N	N	N	N
75	88	N	N	N	N	N
99	96	N	N	N	N	N

where:

NS refers to normal serum (i.e. NS L11 = normal line 11 serum)
Titer refers to the last positive serial dilution of the normal serum prior to addition into the wells

Table 13

Room Temperature Cytotoxicity Testing with Mouse Anti-Chicken T-Cell Monoclonal Antibodies

		Antiserum #																									
Species	Bird	C1	C2	C3	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Quail	1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	6	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	7	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	8	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	10	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	11	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	12	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	13	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Chicken	15	N	N	N	+	+	+	+	N	N	N	+	+	N	N	+	N	N	+	+	+	+	+	+	N	N	+	+
	17	N	N	N	+	+	+	+	N	N	N	+	+	N	N	+	N	N	+	+	+	+	+	+	N	N	+	+
	20	N	N	N	+	+	+	+	N	N	N	+	+	N	N	+	N	N	+	+	+	+	+	+	N	N	+	+

where:
+ indicates a positive reaction
N indicates a negative reaction
C1 contains cells only
C2 contains cells with antiserum
C3 contains cells with rabbit complement
Birds are identified in Appendix 5
Antiserum # designate particular monoclonal antibodies and are listed in Appendix 6

Table 14

RT Cytotoxicity Testing with Mouse Anti-Chicken

RBC B Blood Group Monoclonal Antibodies

Species	Bird no.	C_1	C_2	C_3	Antiserum # (#25)	(#26)	(#27)	(#28)	(#29)
Quail	3	N	N	N	N	N	N	N	N
	4	N	N	N	N	N	N	N	N
	8	N	N	N	N	N	N	N	N
	9	N	N	N	N	N	N	N	N
Chicken	14	N	N	N	N	N	N	N	N
	16	N	N	N	N	N	N	N	N
	18	N	N	N	N	N	N	N	N
	19	N	N	N	N	N	N	N	N
	21	N	N	N	N	N	N	N	N

where:

C_1 consists of cells in RPMI
C_2 consists of cells with antiserum
C_3 consists of cells with rabbit complement

Table 15

37°C Cytotoxicity Testing with Mouse Anti-Chicken

RBC B Blood Group Monoclonal Antibodies

Species	Bird no.	C_1	C_2	C_3	Antiserum # (#25)	(#26)	(#27)	(#28)	(#29)
Quail	3	N	N	N	N	N	N	N	N
	4	N	N	N	N	N	N	N	N
	8	N	N	N	N	N	N	N	N
	9	N	N	N	N	N	N	N	N
Chicken	14	N	N	N	N	N	N	N	N
	16	N	N	N	N	N	N	N	N
	18	N	N	N	N	N	N	N	N
	19	N	N	N	N	N	N	N	N
	21	N	N	N	N	N	N	N	N

where:

C_1 consists of cells in RPMI
C_2 consists of cells with antiserum
C_3 consists of cells with rabbit complement

Figure 1. A Positive Cytotoxicity Score.

A positive cytotoxicity score is shown as seen by the uptake of eosin by the lymphocytes because they have lysed.

Magnification 400X

Figure 2. A Negative Cytotoxicity Score.

Living cells remain intact and unstained.

Magnification 400 X

Figure 3. Negatively and Positively Scored Cells.

Both stained and unstained cells are shown for easier comparison. Positive scoring cells are darkly stained and larger than negative unstained cells.

Magnification 400 X

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VII. Appendix 1

Coturnix coturnix japonica Identification Key

Birds used in Lectin-RBC agglutination

no.	Line	Tag no.	Sex
1	11	14140/14141	M
2		14275/14276	M
3		14392/14393	F
4		14878/14920	M
5		16524/16527	M
6		16813/16814	M
7		16990/16991	F
8		19352/19353	M
9		19370/19371	M
10		19382/19383	M
11		19934/19935	M
12		20111/20112	M
13		20199/20200	F
14		20201/20202	M
15		20335/20336	M
16		20617/20618	F
17		20677/20678	F
18		20773/20774	F
19		20791/20792	F
20		20793/20794	F
21		22057/22058	F
22		22072/22073	M
23		22076/22077	M
24		22567/22568	M
25		22569/22570	M
26		22797/22798	F
27		24040/24041	M
28		24145/24146	M
29		24326/24327	M
30		24407/24408	M
31		24567/24568	M
32		24605/24606	M
33		24926/24927	F
34		28264/28265	M
35		28452/28453	F
36		29027/29028	M
37		29464/29465	M
38		31230/31231	M
39		33902/33903	F

40		35491/35492	F
41		35795/35796	F
42		35976/35977	M
43	27	14582/14583	M
44		14596/14597	M
45		14624/14625	M
46		15156/15157	M
47		15220/15221	M
48		15226/15227	F
49		16737/16738	M
50		19168/19169	M
51		19238/19239	F
52		19318/19319	M
53		19612/19613	M
54		20059/20060	M
55		20301/20302	M
56		20403/20404	M
57		20625/20626	M
58		20627/20628	F
59		20629/20630	F
60		20775/20776	M
61		20777/20778	M
62		20899/20900	M
63		20984/20985	F
64		22045/22046	M
65		22101/22102	M
66		22131/22132	F
67		22174/22175	F
68		22194/22195	F
69		24195/24196	F
70		31953/31954	F
71		33001/33400	M
72		33273/33274	F
73		33577/33578	F
74		35057/35058	M
75		35441/35442	F
76		35763/35764	F
77		35918/35919	M
78		35990/35991	M
79		35986/35987	F
80		36110/36111	F
81		36122/36123	M
82	68	25691/25692	F
83		25693/25694	M
84		25695/25696	F
85		25697/25698	M
86	70	23537/23538	F

87		25346/25347	F
88		25382/25383	M
89		25410/25411	M
90		25418/25419	F
91		25472/25473	M
92		25474/25475	M
93		25619/25620	F
94	71	21830/21831	F
95		21881/21882	M
96		25023/25024	F
97		25147/25148	M
98		30145/30146	M
99		30215/30216	M
100		30219/30220	M
101		30241/30242	F
102		30337/30339	F
103		30339/30340	M
104		30367/30368	M
105		30402/30403	M
106		30647/30648	M
107		30649/30650	F
108		30651/30652	M
109		30653/30654	F
110		30697/30698	F
111		30701/30702	F
112		30840/30841	M
113		30856/30857	F
114		30860/30861	M
115		30862/30863	F
116		30888/30889	M
117		34106/34107	M
118		34175/34176	M
119		34177/34178	M
120		34179/34180	F
121		34181/34182	F
122		34185/34186	M
123		34239/34240	M
124		34241/34242	M
125		34303/34304	F
126	75	31595/31596	M
127		31601/31602	M
128		31603/31604	M
129		31680/33135	M
130		33023/33024	M
131		33027/33028	M
132		33163/33164	M
133		33410/33411	M
134		33436/33437	M
135		33663/33664	M

136	33729/33730	M
137	33839/33840	M
138	33883/33884	F
139	33886/33887	M
140	35020/35021	F
141	35101/35102	F
142	35307/35308	F
143	35339/35340	M
144	35661/35662	M
145	36901/36902	M

VIII. Appendix 2

Coturnix coturnix japonica Identification Key

Birds used in Hemolysis and Cytotoxicity

no.	Line	Tag no.	Sex
1	11	20217/20218	M
2		22115/22116	M
3		22691/22692	M
4		22693/22694	M
5		24040/24041	M
6		24565/24566	M
7		24878/24879	M
8		28264/28265	M
9		28594/28595	M
10		28602/28618	M
11		29016/29544	M
12		29093/29094	M
13		31433/31434	M
14		33221/33222	M
15		35036/35037	M
16		35489/35490	M
17	27	30557/30558	M
18		31895/35555	M
19		33273/33274	F
20		33922/33923	M
21		35057/35058	M
22		35176/35177	M
23		35178/35179	M
24		35519/35520	F
25		35559/35560	M
26		35657/35658	M
27		35763/35764	F
28		35571/35772	M
29		35918/35919	M
30		35958/35959	M
31		35986/35987	F
32		35990/35991	M
33		36110/36111	F
34		36122/36123	M
35		36248/36249	M

36	71	30203/30204	F
37		32531/32532	M
38		32533/32534	F
39		32551/32552	M
40		32557/32558	M
41		32565/32566	M
42		32567/32568	M
43		32569/32570	M
44		32609/32610	M
45		32731/32732	M
46		32743/32744	M
47		32879/32880	F
48		34181/34182	F
49		34902/34903	M
50		34904/34905	M
51		34944/34945	M
52		37187/37188	M
53		37189/37190	M
54		37249/37250	M
55		37325/37326	M
56		39134/39135	M
57		39178/39179	M
58		39307/39308	M
59		39349/39350	M
60		39361/39362	M
61		39385/39386	M
62		39449/39450	M
63		39457/39458	M
64		39596/39597	M
65		39672/39673	M
66		39718/39719	M
67		39726/39727	M
68		40223/40224	M
69		40352/41934	M
70		41569/41570	M
71		41740/41741	M
72	75	32997/32998	M
73		36865/36866	M
74		38318/38319	M
75		38494/39495	M
76		38496/38497	M
77		38502/38503	M
78		38584/38585	M
79		38586/38587	M
80		38725/38726	M
81		38747/38748	M
82		38822/38823	M
83		38858/38859	M
84		38934/38935	M
85		38953/38954	M
86		39062/39063	M

87		39064/39065	M
88		39066/39067	M
89	99	40242/41997	M
90		40243/41941	M
91		40249/41628	M
92		40301/41664	M
93		41612/41614	M
94		41616/41617	M
95		41638/41639	M
96		41642/41644	M
97		41693/41696	M
98		41856/41858	M
99		41923/41924	M

IX. Appendix 3

Table of Alloantisera Specificities and Sources

Serum	Antiserum specificity	Line source	Bird no.
#1	Anti 11	71	49 50 51 52 53 54 55
#2X	Anti 27 (7-9 months)	71	58 59 60 62 63 65
#2Y	Anti 27 (15 months)	71	58 59 60 62 63 65
#3	Anti 71	75	75 76 77 79 81 82 85
#4	Anti 75	71	56 57 61 64 66

67

#5	Normal Anti 11	11	13
#6	Normal Anti 11	11	10
#7	RBC absorbed Anti 11	27 71 75	35 46 87
# 8	RBC absorbed Anti 11	27 71 75	18 36 72
#9	RBC & Lymphocyte absorbed Anti 11	27 71 75	18 36 72
#10	Whole Blood absorbed Anti 11	27 71 75	20 43 74
#11	RBC absorbed Anti 11	11	6
#12	Normal Anti 27	27	17
#13	Normal Anti 27	27	27
#14	Normal Anti 27	27	26
#15	RBC absorbed Anti 27	11 71 75	1 46 87
#16	RBC absorbed Anti 27	11 71 75	11 36 72
#17	RBC & Lymphocyte	11	11

	absorbed Anti 27	71	36
		75	72
#18	Whole Blood	11	5
	absorbed Anti 27	71	43
		75	74
#19	RBC absorbed	27	30
	Anti 27		
#20	Normal Anti 71	71	41
#21	Normal Anti 71	71	40
#22	Normal Anti 71	71	48
#23	RBC absorbed	11	1
	Anti 71	27	35
		75	87
#24	RBC absorbed	11	11
	Anti 71	27	18
		75	72
#25	RBC & Lymphocyte	11	11
	absorbed Anti 71	27	18
		75	72
#26	Whole Blood	11	5
	absorbed Anti 71	27	20
		75	74
#27	RBC absorbed	71	37
	Anti 71		
#28	Normal Anti 75	75	81
#29	Normal Anti 75	75	72

#30	Normal Anti 75	75	80
#31	RBC absorbed Anti 75	11	1
		27	35
		71	46
#32	RBC absorbed Anti 75	11	11
		27	18
		71	36
#33	RBC & Lymphocyte absorbed Anti 75	11	11
		27	18
		71	36
#34	Whole Blood absorbed Anti 75	11	5
		27	20
		71	43
#35	RBC absorbed Anti 75	75	84
#36	Whole Blood absorbed Anti 27 (15 months)	11	3
		71	47
		75	86
#37	Normal Anti 11	11	11
#38	Normal Anti 27	27	31
#39	Normal Anti 71	71	42
#40	Normal Anti 75	75	72
#41	Normal Anti 99	99	94
#42	Anti 99	71	68
			69
			70
			71

#43	Anti 71	99	90
			92
			93
			98
#44	Whole Blood	27	29
	absorbed Anti 99	71	47
		75	86
#45	Whole Blood	11	10
	absorbed Anti 99	27	20
		71	44
		75	87
#46	Whole Blood	11	9
	absorbed Anti 99	27	23
		71	46
		75	73
#47	Whole Blood	27	29
	absorbed Anti 71	75	86
		99	97
#48	Whole Blood	11	10
	absorbed Anti 71	27	20
		75	87
		99	89
#49	Whole Blood	11	9
	absorbed Anti 71	27	23
		75	73
		99	95

X. Appendix 4

Rapid Cytotoxicity Test

An Alernate Method to Conventional Cytotoxicity Tests

The rapid cytotoxicity test (Mittal et al '69) was modified from a conventional cytotoxicity test (Terasaki et al '67). By shortening the time required to isolate lymphocytes, and shortening the incubation periods, and accelerating the scoring time, a 45 minute cytotoxicity test was developed. These modifications and the possibilities of extending them to the cytotoxicity test used in the present study follow.

Human lymphocytes are isolated in approximately 10 minutes in the rapid test by the following method. Blood samples are centrifuged at 5000g for 2 minutes. The buffy coat is removed with a small amount of supernatant plasma, treated with anti A, anti B, or anti H antisera to agglutinate RBC's, and placed on glass beads contained in a plastic drinking straw. The beads are washed first with 37°C saline, then with 37°C HPBS. The straw is placed in a 37°C water bath for 10 minutes after being stoppered. Polymorphonuclear leukocytes stick to the beads and granulocytes adhere to a nylon sieve contained at the bottom of the straw. Lymphocytes are separated from agglutinated RBC's by slow (2 second) centrifugation. The lymphocytes are suspended in HPBS.

It may be possible to employ this method of lymphocyte isolation for quail lymphocytes since alloantiserum has been prepared against each line so that RBC's may be agglutinated by specific alloantiserum (i.e. L 11 RBC's agglutinated with anti 11 antiserum). However, the isolated lymphocytes may become coated with alloantibodies and this may contaminate a test (especially if testing lymphocytes for cross-reactivity with antiserum from another line). It remains to be seen if this isolation method is efficient and as rapid as the Ficoll-hypaque technique and whether it will permit modification of the conditions. The test proceedure may require modification to room temperature or below because of the poor 'in vitro' survival of quail lymphocytes at 37°C.

The conditions of the rapid test also require the enzymatic treatment of the lymphocytes with a 5% ficin solution in PBS at 37°C for 5 minutes, dilution with HPBS, centrifuging for 1 minute at 5000g, washing, and suspension in McCoy's medium. Ficin treatment makes the test more sensitive and the incubation time of the antiserum, lymphocytes, and complement can be reduced 3 fold to 15-20 minutes. The result of the ficin treatment is that lymphocytes and antiserum need only be incubated for 10 minutes and an additional 10 minutes after the complement is added. Mittal et al ('69) have suggested that this 20 minute incubation may be further reduced to 10 minutes by adding lymphocytes, antiserum, and complement all at once. This modification may pose a problem to the test used in the

present study. The ficin treatment may have to be modified to room temperature or below.

Scoring time is greatly reduced by attaching an electric typewriter to the microscope. This permits the examination of 120 antisera in 5 minutes. The time required to score the same number of antisera in the present study is 1 hour. This last modification is a simple mechanical one and should pose no problem for modification of the present technique.

XI. Appendix 5

Coturnix coturnix japonica Identification Key

Birds Used in Cytotoxicity Tests with Monoclonal Antibodies

no.	Species	Line or B Specificity	Tag no.	Sex
1	Quail	11	22115/22116	M
2			24565/24566	M
3			35036/35037	M
4		27	31895/35555	M
5			35377/35378	M
6			39339/39340	M
7		71	32567/32568	M
8			32743/32744	M
9		75	36865/36866	M
10			39060/39061	F
11			39066/39067	M
12		99	41616/41417	M
13			41970/41971	F
14	Chicken	2/2	33227/33228	F
15		2/2	33231/33232	M
16		14/14	33329/33330	M
17		15/15	32868/32869	M
18		15/15	32994/BB141	M
19		19/19	33574/33575	M
20		21/21	32899/32900	M
21		21/21	33361/33362	M

Appendix 6

Mouse Anti-Chicken Monoclonal Antibody Specificity and Source

Serum	MRC Designation	Specificity	Source
#1	24C8	Anti-Chicken Lymphocyte	Ascites supernatant
#2	24C30	Anti-Chicken Lymphocyte	Ascites supernatant
#3	24C31	Anti-Chicken Lymphocyte	Ascites supernatant
#4	24C35	Anti-Chicken Lymphocyte	Ascites supernatant
#5	25C8	Anti-Chicken Lymphocyte	Culture Medium supernatant
#6	25C18	Anti-Chicken Lymphocyte	Culture Medium supernatant
#7	25C22	Anti-Chicken Lymphocyte	Culture Medium supernatant
#8	25C23	Anti-Chicken Lymphocyte	Culture Medium supernatant
#9	26C1	Anti-Chicken Lymphocyte	Culture Medium supernatant
#10	26C7	Anti-Chicken Lymphocyte	Culture Medium supernatant
#11	26C8	Anti-Chicken Lymphocyte	Culture Medium supernatant
#12	26C10	Anti-Chicken Lymphocyte	Culture Medium supernatant
#13	26C20	Anti-Chicken Lymphocyte	Culture Medium supernatant
#14	26C21	Anti-Chicken Lymphocyte	Culture Medium supernatant
#15	26C22	Anti-Chicken Lymphocyte	Culture Medium supernatant
#16	26C23	Anti-Chicken Lymphocyte	Culture Medium supernatant
#17	26C27	Anti-Chicken Lymphocyte	Culture Medium supernatant
#18	26C34	Anti-Chicken Lymphocyte	Culture Medium supernatant
#19	26C35	Anti-Chicken Lymphocyte	Culture Medium supernatant
#20	26C36	Anti-Chicken Lymphocyte	Culture Medium supernatant
#21	26C39	Anti-Chicken Lymphocyte	Culture Medium supernatant
#22	26C41	Anti-Chicken Lymphocyte	Culture Medium supernatant
#23	26C43	Anti-Chicken Lymphocyte	Culture Medium supernatant
#24	26C45	Anti-Chicken Lymphocyte	Culture Medium supernatant
#25	Ch-5	Anti-Chicken RBC 2/2	Culture Medium supernatant
#26	Ch-62	Anti-Chicken RBC 14/14	Culture Medium supernatant
#27	Ch-45	Anti-Chicken RBC 15/15	Culture Medium supernatant
#28	Ch-32	Anti-Chicken RBC 19/19	Culture Medium supernatant
#29	LC-5	Anti-Chicken RBC 21/21	Culture Medium supernatant

*(Longenecker & Mosmann '82)

CANADIAN THESES

THESES CANADIENNES

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